Nutrient-Based Chemical Library as a Source of Energy Metabolism Modulators

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Supporting Information Placeholder

ABSTRACT: Covalent conjugates of multiple nutrients often exhibit greater biological activities than each individual nutrient and more predictable safety profiles than completely unnatural chemical entities. Here we report the construction and application of a focused chemical library of 308 covalent conjugates of a variety of small-molecule nutrients. Screening of the library with a reporter gene of sterol regulatory element-binding protein (SREBP), a master regulator of mammalian lipogenesis, led to the discovery of a conjugate of docosahexaenoic acid (DHA), glucosamine, and amino acids as an inhibitor of SREBP (molecule **1**, DHG). Mechanistic analyses indicate that molecule **1** impairs the SREBP activity by inhibiting glucose transporters and thereby activating AMP-activated protein kinase (AMPK). Oral administration of molecule **1** suppressed the intestinal absorption of glucose in mice. These results suggest that such synthetic libraries of nutrient conjugates serve as a source of novel chemical tools and pharmaceutical seeds that modulate energy metabolism.

INTRODUCTION

Carbohydrates, lipids, amino acids, and vitamins constitute some of the nutrients essential for the life of an organism. On the other hand, there exist a number of pharmaceuticals and functional foods that consist of covalently conjugated multiple nutrients. For example, tocopherol nicotinate, a clinically used lipid antioxidant (Figure S1A),[1] is a synthetic conjugate of vitamins E and B_3. Another example is a conjugate of vitamin C and glucose, AA-2G (Figure S1B).[2] This long-acting prodrug, used as a component of cosmetics and supplements, is enzymatically metabolized to gradually release vitamin C and glucose. The nutrient conjugates exemplified by these two examples tend to show greater potency than a mixture of the individual components and more predictable safety profiles than completely unnatural chemical entities.

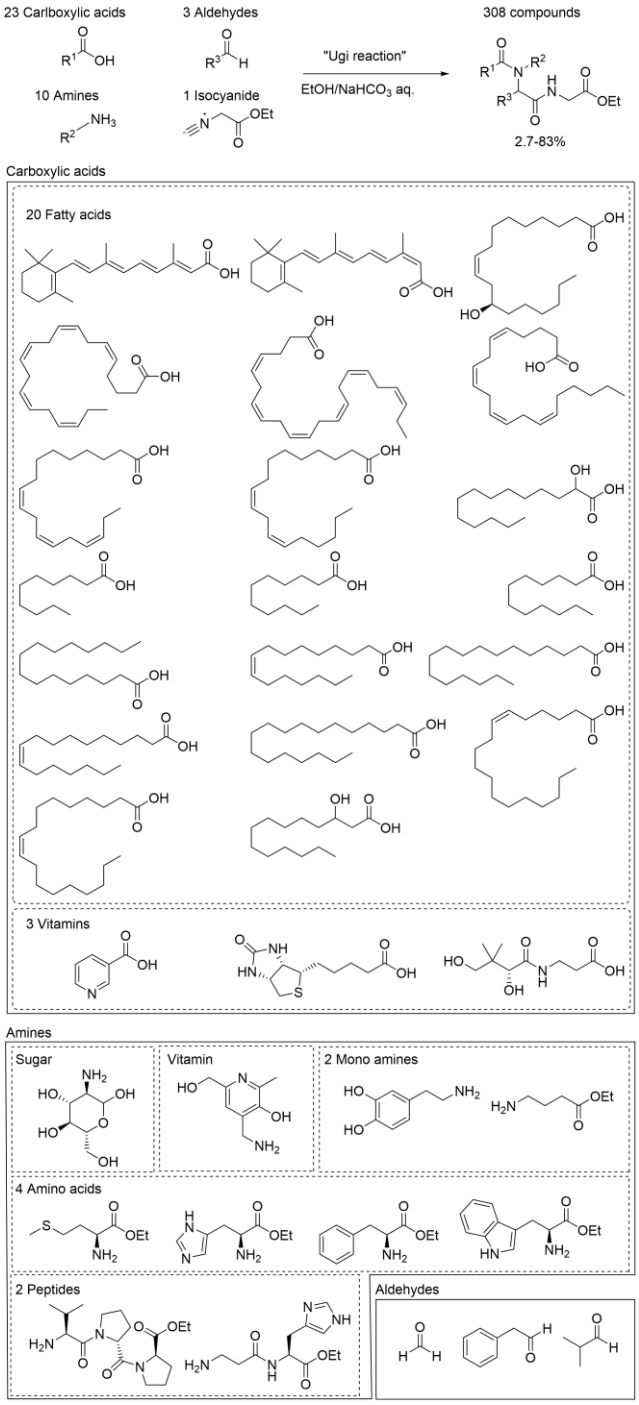
A large number of nutrient conjugates are also naturally occurring in organisms. For example, NA-Gly, a conjugate of arachidonic acid and glycine (Figure S1C), plays distinct roles from those of the parental nutrients, including inhibition of T-type calcium channel and fatty acid amide hydrolase.[3] These considerations led to the hypothesis that combinatorial chemical synthesis of covalent nutrient conjugates produces a focused chemical library that potentially contain unique, relatively safe biologically active molecules.

RESULTS AND DISCUSSION

To construct a library of nutrient conjugates, we employed Ugi reaction, a four-component reaction involving a ketone or aldehyde, an amine, an isocyanide, and a carboxylic acid to form a bis-amide[4] (Scheme 1). The use of Ugi reaction for chemical library construction is not new: a number of other studies have generated chemical libraries through the multi-component one-pot reaction.[5,6] Nevertheless, it has never been used for constructing a library of nutrient conjugates. Reaction components were chosen mainly from nutrients or their precursors that are often included in multivitamin supplements: 20 fatty acids and 3 vitamins as carboxylic acid components; glucosamine and a vitamin, two monoamines, 4 amino acids, and two biologically active peptides (VPP[7] and carnosine[8,9]) as amine components, 3 alde-

hydes corresponding to glycine, valine, phenylalanine; and ethyl isocyanoacetate to form a glycine ester terminus. Combinatorial parallel Ugi reactions of these components yielded 308 nutrient conjugates. Purities of the desired products were checked by LC-MS, and reaction yields ranged from 2.7-83% (Table S1).

Scheme 1. Construction of Nutrient Oriented Chemical Library



One biological activity expected from these nutrient conjugates was the modulation of energy metabolism in mammalian cells. For proof of concept, we screened the library by using a reporter gene of sterol regulatory element-binding protein (SREBP), a master transcription factor of lipogenesis.[10] SREBP is positioned in the far downstream of mammalian energy metabolism pathways,[11] so that the SREBP reporter gene activity serves as an indicator for a myriad of cellular metabolic alterations. Chinese hamster ovary (CHO)-K1 cells were transfected with the SREBP reporter gene in which the expression of firefly luciferase is controlled by three SREBP binding sites. A constitutively active β-Gal reporter gene was also co-transfected for normalization purposes.[12] We conducted initial screening at 10 μM of the compounds and selected screening hits that exhibited SREBP inhibition higher than that of 25-hydoroxycholesterol (HC), a known endogenous inhibitor of SREBP, after 24-h incubation (Table S1). Repeated experiments identified 5 conjugates that showed clear dose-dependence (Figure S2). Of the five conjugates, three molecules were structurally analogous (Figure S2), and the most potent molecule was molecule **1**, which is composed of DHA, glucosamine, and phenylalanine-glycine dipeptide (Figure 1A). We therefore focused our efforts on molecule **1** (also referred to as DHG) for further investigations.

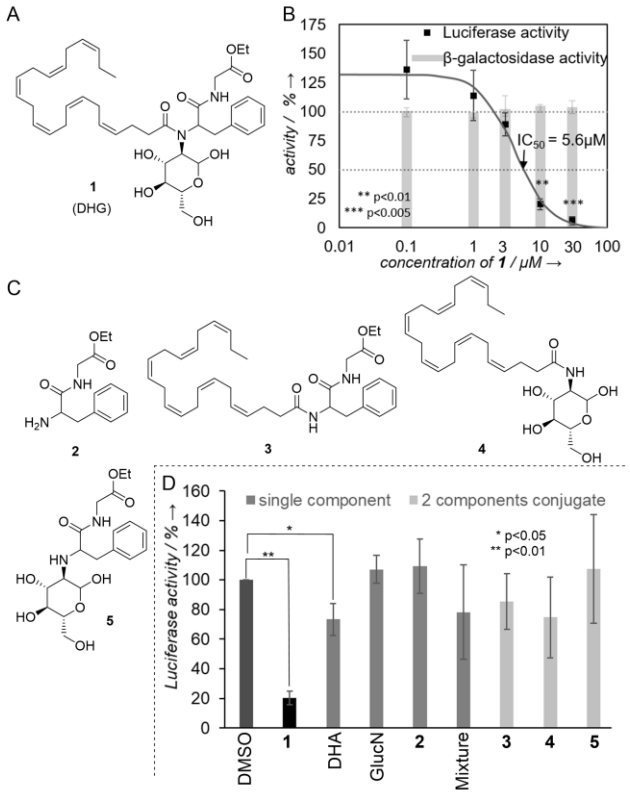


Figure 1. Chemical structure of molecule **1** and its SREBP inhibitory activity. (A) Chemical structure of molecule **1** (DHG). (B) Dose response curve for molecule **1**. Molecule **1** suppressed the ability of endogenous SREBPs to activate transcription of a luciferase reporter gene. The IC_{50} value was estimated to be 5.6 μM from a four-parameter logistic model fitting curve (Image J). The β-galactosidase activity showed no detectable change in the tested concentrations of molecule **1**. (C) Chemical structures of dipeptide **2** and two-components conjugates (**3**-**5**). (D) SREBP inhibitory activities of molecules **1**-**5**. Luciferase activities were measured at a concentration of 10 μM after 24-h incubation. Each single component (DHA, glucosamine [GlucN], and dipeptide **2**) or their mixture exhibited weaker activity than molecule **1**. The two-components conjugates (**3**-**5**) also displayed weaker activities than molecule **1**.

The IC_{50} value of molecule **1** for the SREBP reporter gene was estimated to be 5.6 μM, while its effects on the β-Gal reporter gene up to 30 μM were undetectable, indicating that 24-hour incubation with molecule **1** has no detectable cytotoxicity up to 30 μM (Figure 1B). We examined the ability of each of the components (DHA, glucosamine, and phenylalanine-glycine dipeptide **2**) to inhibit SREBP at 10 μM (Figure 1D). Although DHA was found to be slightly active, as previously reported,[13] the other two were essentially inactive. Importantly, the activity of a mixture of the three components was as low as that of DHA. We chemically synthesized three partial structures of molecule **1** by combining two of the components (molecules **3**-**5**) (Figure 1C). Although molecules **3** and **5**, which contain DHA, exhibited activity similar to that of DHA, the partial structures failed to exert clear inhibitory activity at 10 μM (Figure 1D). These results indicate that covalent conjugation of all the three components is required for the potent activity of molecule **1**.

To gain insights into the mechanism of action, we examined the status of an SREBP protein upon treatment with molecule **1**. Newly synthesized SREBPs are localized on the endoplasmic reticulum (ER) membrane, where they bind to SREBP cleavage-activating protein (SCAP), an escort protein of SREBPs.[14,15] At low levels of cellular sterol, the SREBP-SCAP complex is transported from the ER to the Golgi apparatus, where SREBPs are cleaved sequentially by site-1 protease (S1P) and site-2 protease (S2P), liberating the NH_2-terminal transcription factor domain of SREBPs.[10,16] The mature form translocates to the nucleus, where it activates lipogenic genes.[10] The activation of SREBPs is tightly regulated by a negative feedback loop in which cholesterol and 25-hydroxycholesterol (25-HC) bind to SCAP and Insigs (ER anchor proteins), respectively, resulting in retention of the SREBP-SCAP complex in the ER.[17,18] Western blot analysis of SREBP-2 in CHO-K1 cells indicates that molecule **1** decreases the amounts of the mature form and accumulates the precursor form until 6 h of incubation, much like 25-HC (Figure 2A). However, prolonged treatment with molecule **1** decreased the amounts of the precursor, whereas 25-HC did not (Figure 2A). Since SREBP proteins are unstable once released from SCAP,[19,20,21] molecule **1** is likely to inhibit the activation process of SREBP-2 posterior to its release from SCAP unlike 25-HC does.

The relatively large structure of molecule **1** (molecular weight: 722.92) prompted us to estimate its membrane permeability. Parallel artificial membrane permeability assay (PAMPA) showed a limited permeability of molecule **1** through a lipid-infused artificial membrane (Mean Papp: 1.19 x 10^{-6} cm/s). The low membrane permeability increased the possibility that molecule **1** either targets extracellular proteins or is actively transported into cells. A number of studies have shown that covalent conjugations of nutrients and vitamins increases active transport of pharmaceuticals.[22,23]

The presence of a glucose unit in molecule **1** encouraged us to examine the effects of glucose transporter inhibitors on the activity of molecule **1**. Glucose is known to be transported by two classes of transporters: glucose transporters (GLUTs) or sodium-glucose transport proteins (SGLTs). To our surprise, western blot analyses of SREBP-2 in CHO-K1 cells revealed that co-incubation with phloretin, a representative GLUT inhibitor, *enhanced* the ability of molecule **1** to decrease the protein levels of SREBP-2, while that with phlorizin, a representative SGLT inhibitor, failed to do so (Figure 2B). Importantly, phloretin alone, but not phlorizin, decreased the amounts of both the mature and precursor forms of SREBP-2 in CHO-K1 cells, although not as potently as molecule **1** did (Figure 2B). These results imply that molecule **1** impairs SREBP-2 activation by inhibiting GLUTs rather than by being transported through GLUTs.

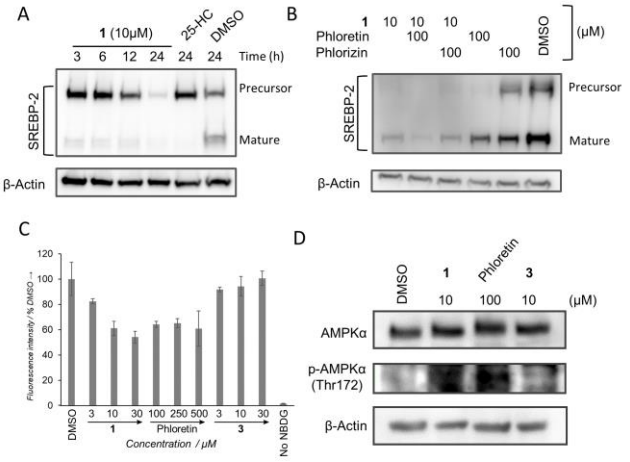


Figure 2. Mechanistic analysis of molecule **1** (A) Effects of molecule **1** on the SREBP-2 maturation. CHO-K1 cells were treated with molecule **1** (10 μM) or 25-hydroxycholesterol (5 μM) for varied lengths of time, and the cell lysates were analyzed by western blots with an SREBP-2 antibody. Positions of precursor and mature forms of SREBP-2 are indicated on the right. (B) Effects of transporter inhibitors on the SREBP-2 maturation. Glucose transporter (GLUT) inhibitor phloretin (100 μM) or sodium-glucose transport protein (SGLT) inhibitor phlorizin (100 μM) were added to the culture medium of CHO-K1 cells in the presence or absence of molecule 1 (10 μM). After 24-hour incubation, the cell lysates were analyzed by western blots with an SREBP-2 antibody. Positions of precursor and mature forms of SREBP-2 are indicated on the right. (C) Inhibition of cellular glucose uptake by molecule 1. Uptake of fluorescently-labeled glucose analog 2-NBDG was measured with human Caco-2 cells. The cells were treated simultaneously with NBDG (200 μM) and varied concentrations of molecule **1** (3-30 μM), molecule 3 (3-30 μM), or positive control phloretin (100-500 μM). After extensive wash, fluorescence of the cells was measured with a fluorescence plate reader (excitation: 490 nm; emission: 530 nm). (D) AMPK activation by molecule **1**. Hepa 1-6 cells were treated with molecule **1** (10 μM), phloretin (100 μM), or molecule **3** (10 μM), and the cell lysates were analyzed by western blots with an antibody specific for phosphor-Thr172 AMPK. It is evident that molecule **1** activates AMPK phosphorylation just like phloretin.

To support the hypothesis, we next examined the ability of molecule **1** to block glucose uptake of differentiated Caco-2 cells, a human intestinal cell line that expresses GLUTs, especially GLUT2[24] Molecule **1** blocked cellular uptake of the fluorescently-labeled glucose analog 2-NBDG (2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose)[25,26] in a dose-dependent manner, whereas a control molecule that lacks the sugar moiety of molecule **1** (molecule **3**) had little effects. No apparent cell death was observed during the course of the 3-hour study. The inhibitory activity of mole-

cule **1** at 10 μM was comparable to that of high concentrations of phloretin (Figure 2C).

There are four major glucose transporters in human cells: GLUT1-4 (SLC2A1-4). Overexpression of each glucose transporter increased glucose uptake in HEK293 cells, and the ability of molecule **1** to cancel the increase was examined. The results (Figure S3) suggest that molecule **1** exhibits broad selectivity to glucose transporters. Stronger inhibition was observed for GLUT2-4 than for GLUT1, which is consistent with the results of Caco-2 cells whose major glucose transporter is GLUT2.[24,27]

Inhibition of cellular glucose uptake usually results in a shortage of intracellular energy, leading to the activation of AMP-activated protein kinase (AMPK), a sensor of cellular energy and nutrient status.[28,29] It has also been demonstrated that the activation of AMPK induces phosphorylation of SREBPs to block the nuclear translocation of SREBPs, so that the cells terminate lipogenesis upon limited availability of cellular energy.[30] Western blot analyses with an antibody against phospho-AMPK indicate that 3-hour treatment of Hepa1-6 cells with molecule **1** (10 μM) induced the Thr172 phosphorylation of AMPK as much as that with phloretin (100 μM) did, whereas three partial structures of molecule **1** (molecules **3-5**) failed to induce the phosphorylation of AMPK (Figure 2D and Figure S4). At this moment, we cannot rule out the possibility that molecule **1** has SREBP-inhibition mechanisms other than the energy depleting activity. Nonetheless, the inhibition of glucose transporters and the subsequent activation of AMPK provides one possible explanation for the observed SREBP inhibition in the cultured cells.

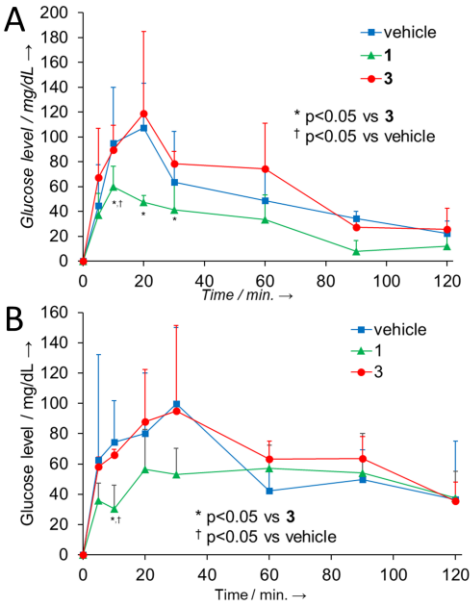


Figure 3. Glucose uptake in fasted mice. (A) Oral co-administration of molecule **1** and glucose. Molecule **1** (6.25 μmol) or molecule **3** (6.25 μmol) was orally co-administrated into fasted mice (n=3) with glucose (12.5 mg) in water (250 μL). (B) Pretreatment with molecule **1** prior to glucose oral administration. Molecule **1** (6.25 μmol) or molecule **3** (6.25 μmol) was orally administrated into fasted mice (n=3) 10-min before oral administration of glucose (12.5 mg) in water (250 μL). Blood samples were collected at indicated time points and analyzed by a blood glucose self-monitoring kit.

The glucose uptake inhibitory activity in Caco-2 cells and the limited cell permeability of molecule **1** encouraged us to examine the effects of orally administered molecule **1** on intestinal absorption of glucose in mice. Mice were fasted for 24-hours prior to oral administration of a glucose solution comparable to typical sweetened beverage products. Oral co-administration of molecule **1** (Figure 3A) or its administration 10 min prior to the glucose administration (Figure 3B) significantly suppressed the increase of blood glucose levels. In contrast, molecule **3**, which is devoid of the glucose segment, exhibited no detectable effects. We found no apparent toxicity of molecule **1** in mice such as diarrhea during the course of the experiments. Although the suppression of blood glucose levels may not necessarily due to the inhibition of glucose transporters, these animal effects were consistent with the glucose uptake inhibition observed in the cultured cells.

In the present study, a chemical library of 308 covalent conjugates of multiple nutrients and vitamins led to the discovery of molecule **1** as a new inhibitor of glucose uptake *in vitro* and *in vivo*. It remains unclear how this particular nutrient conjugate blocks glucose uptake. Perhaps the glucose moiety of molecule **1** may be recognized by glucose transporters, but the hydrophobic DHA tail and bulky dipeptide segment could prevent the transport of the entire molecule.

Close inspection of the screening results indicates the two major structural requirements for the activity. First, the molecule **1** analogs with unsaturated fatty acids tend to be more potent than those with saturated fatty acids (Figure S5). Covalent conjugates of unsaturated fatty acids and glycine have been reported to block glycine transporters.[31]Although it remains unknown why unsaturated fatty acids work better than saturated fatty acids, covalent conjugation of unsaturated fatty acids with nutrients might provide a general strategy to designing nutrient transporter inhibitors.

Second, replacement of the phenylalanine segment with glycine or valine impairs the activity (Figure S6). A number of glucose transporter inhibitors have been documented in the literature.[32] One recently reported GLUT1 inhibitor has a peptidic structure containing a fluorinated phenylalanine component and two other phenyl groups, all of which interact extensively with the transporter near the glucose-binding poket.[33] The phenylalanine segment of molecule **1** might similarly assist the interaction of molecule **1** in the glucose-binding pocket.

In conclusion, we report the construction of a focused chemical library of 308 covalent conjugates of a variety of small-molecule nutrients. The utility of the library was demonstrated by isolation of new SREBP inhibitors that target glucose transporters. The nutrient conjugates are expected to exert a myriad of biological activities due to the fact that each nutrient component *per se* plays certain physiological roles. On the other hand, it also means that such a conjugate might display multiple distinct activities. We checked whether molecule **1** modulates the activity of LXR, a nuclear receptor that controls the expression of SREBP in response to a number of nutrients including 25-HC and glucose.[34,35] Reporter gene assays indicate that molecule **1** exhibited no significant effects on LXR (Figure S7). Effects of molecule **1** on the enzymatic activity of hexokinase, another glucose-binding protein, were also examined *in vitro* and found to be negligible (Figure S8). Although we cannot conclude

that molecule **1** is truly specific to glucose transporters, such covalent conjugations modulate the selectivity of the nutrients to their original targets. Expansion of nutrient-based chemical libraries would provide invaluable source of novel chemical tools and pharmaceutical seeds that modulate energy metabolism.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: @@@. The PDF file includes details of experimental methods and supporting figures (PDF).

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Notes

The authors declare no competing financial interests.

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